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INTRODUCTION

NF1 is characterized by the appearance of multiple tumors of the peripheral nerve, and occasionally the malignant transformation of these tumors. The only available but unsatisfying therapy is surgical tumor resection. The purpose of this study is the preclinical testing of multiple available tyrosine kinase inhibitors for NF1-associated MPNST using *in vitro* and *in vivo* systems. Test systems are to be established. Molecular analysis of the targeted receptor tyrosine kinases will show the incidence of alterations in the receptors and provide a profile of activation of the associated pathways in MPNST. According to the patterns of receptor tyorsine kinase receptor activity appropriate inhibitors will be tested for effects on tumor growth. The findings will substantiate causal therapy attempts based on the tumor specific tyrosine kinase receptor activity profile.

BODY

Task 1

- to establish cell lines from MPNST from NF1 patients

Primary MPNST cell lines were established from 13 tumors. By now, one cell line was established from these primary cultures. This cell line is currently being characterized and tested for identity.
Furthermore, primary cultures were established from 20 PNF tumors.

For xenograft tumor models and for in vitro drug testing, we are using our earlier MPNST-derived cell lines S462 and S805. Any additional cell line will be evaluated for tyrosine kinase activity and used for in vitro / in vivo drug testing depending on this profile.

- recruitment and consenting of patients 20 NF1 patients with MPNST and 20 patients with pNF

13 MPNST and 20 PNF patients with NF1 were recruited. Clinical examination and genetic characterization of patients has been completed. Tumors have been collected and frozen for further mutation analysis (*NF1, TP53* and *INK4A)*. Primary cell cultures were established for all tumors and frozen for further characterization (not shown).

Genetic verification has been completed for the novel MPNST cell line for NF1, TP53 and INK4A. Identity testing is ongoing.

In summary, Patient recruitment is well in time. Primary cells and tumor tissue has been archived and is ready for molecular characterization. Genetic characterization for tumor tissues (NF, TP53, INK4) is complete. One additional MPNST cell line was established.

Task 2

-to determine the profile of PDGFR-α, c-Kit, EGFR and Neu signaling in the MPNST and pNF, and cultured MPNST cells

Protein expression for PDGFR-α, c-Kit, EGFR and Neu was analyzed by immunhistochemistry. It was shown that all MPNST biopsies express high levels of PDGFRA and-B, medium levels of Neu, and moderate to low levels of EGFR and c-Kit (Figure 2, supporting data). PDGFA and -B are also expressed in the tumor tissues (not shown).

The receptor profile was also analyzed in cultured MPNST cell lines (S462, S805), where PDGFRA and –b, Neu and EGFR expression was detected at moderate levels, while c-Kit expression was low (data not shown).
Finally, the receptor profile was also analyzed in xenograft tumors of S462 cells (Figure 1, supporting data) and S805 MPNST cell lines (not shown). In tumors derived from both cell lines, expression of all receptors was high (Figure 1, supporting data).

In summary, the data indicate that a similar profile of tyrosine kinase receptors, along with their ligands, is expressed in MPNST in MPNST derived cell lines and in xenograft tumors derived from these cell lines. Relative expression levels differ between these stages, with highest expression for all receptors in the xenograft tumors and lowest in cell culture.

Task 3

-to test single and multiple (combinations) tyrosine inhibitors *in vitro*

Our pilot data showed an effect of gleevec on MPNST cell line S462 (Holtkamp et al, 2006, appendix). This was confirmed, however, inhibition by gefitinib, an inhibitor of PDGF and c-Kit shows a more potent inhibitory effect on S462 MPNST cell proliferation (Figure 4, supporting data).

In summary, EGFR as well as PDGFR/c-Kit inhibitors reduce proliferation of MPNST tumour cell line S462.

Task 4

- to inhibit tyrosine kinase receptors *in vivo*

MPNST cell lines S462 and S805 were used to grow xenograft tumors in mice (Figure 3, supporting data). In addition, PNF tumor fragments grow tumors in mice when implanted in proximity to the sciatic nerve. These tumors also express PDGFR, EGFR, c-Kit and vascularise (data not shown). Cell line S805 forms very slow growing tumors, therefore, S462 has been selected for further in vivo experiments.

In summary, tumor models have been established for MPNST and PNF.

KEY RESEARCH ACCOMPLISHMENTS

- Establishing of a pool of characterized tumor specimen (MPNST, PNF) together with accompanying primary cell lines and information on their tyrosine kinase receptor profile

- Establishing of a xenograft model for MPNST and PNF suited to test tyrosine kinase receptor inhibitor effects on tumor growth
- Establishing the tyrosine kinase receptor expression in MPNST, PNF biopsies and MPNST and PNF xenografts
- Determination of the effects of the TKR inhibitors Gleevec (EGFR/c-erbB) and Gefitinib (PDGFR/c-Kit) on MPNST cell lines in vitro

REPORTABLE OUTCOMES

Holtkamp et al., Carcinogenesis 2006 (see appendix)

CONCLUSION

Results confirm the expression profile of the TKR PDGFR, EGFR, c-erbB/Neu and c-Kit in all MPNST and PNF tested. The relative expression levels of TKR in the profile differ.

The results also establish a useful tumor model for human MPNST and PNF. In this model, the TKR profile is maintained, but relative expression levels vary clearly from tumor biopsies.

Finally, the TKR inhibitors Gefitinib and Gleevec inhibit proliferation of MPNST derived cell lines.

So what?

The chosen xenograft tumor model reflects the TKR situation in humans only partially. Tumor growth data with TKR inhibitors need to be evaluated carefully in order to not overestimate their potential value.

At least in vitro, the EGFR/c-erbB pathways, inhibited by Gefitinib, appear to more sensitively effect cell proliferation than the PDGF/cKit inhibitor Gleevec. It needs to be shown whether these drugs alone or in combination show synergistic effects in vivo in our xenograft models.

In general, stronger, more selective drugs become available for TKR. We propose to use these drugs, if possible, in this study and replace older drugs. For example, a novel Sanofi-Aventis drug, which is more potent than Gleevec, and more specific with an inhibitory profile for PDGFR, c-Kit and Flt-3, should be tested. Also testable and available is the Novartis drug AMN107, a Kit and PDGFR inhibitor in clinical trial for CML. We propose this change of the SOW to be more flexible in the selection of drugs and in finding the best combination. This change will for now only be applied for the in vitro studies.

REFERENCES

None

APPENDICES

Holtkamp N, Okuducu AF, Mucha J, Afanasieva A, Hartmann C, Atallah I, Estevez-Schwarz L, Mawrin C, Friedrich RE, Mautner VF, von Deimling A. Mutation and expression of PDGFRA and KIT in malignant peripheral nerve sheath tumors, and its implications for imatinib sensitivity. Carcinogenesis. 2006 Mar;27(3):664-71.

SUPPORTING DATA

Figure 1: Tumor xenograft model for MPNST. Human MPNST cells were grafted onto nude mice ($5x10^6$ cells injected, subcutaneously). Tumors were used at 105 days post grafting to detect expression of different tyrosoine kinase receptos (EGFR, c-ErbB, PDGFRA and B, Kit) as well as the proliferation marker Ki67. The data show that tumors remain vital and expression of the tyrosine kinase receptors EGFR, PDGFR and Kit is high. Thus, this model is suitable for testing tyrosine kinase receptor inhibitors for their effect on tumorigenesis. Data shown were obtained using S462 cells. Similar data were obtained with cell line S805.

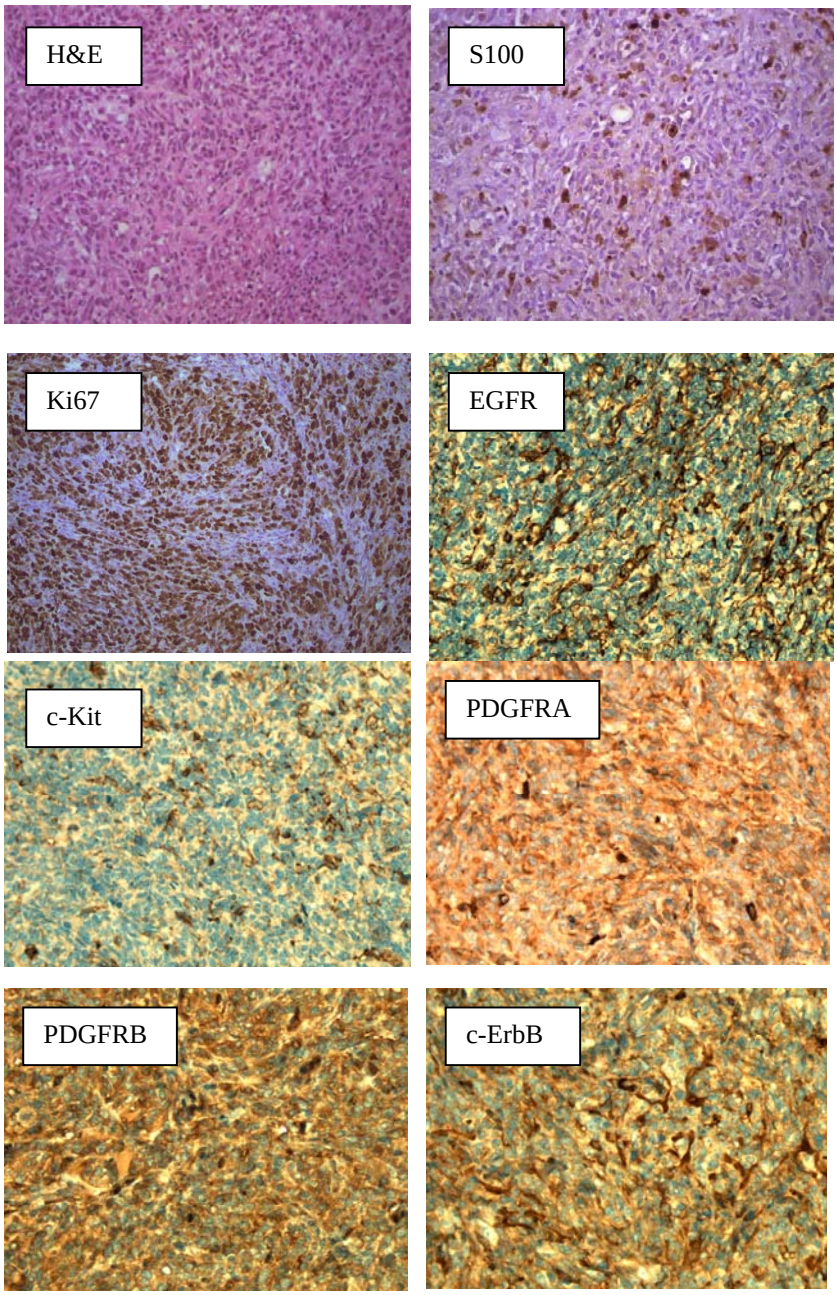


Figure 2: Receptor tyrosine kinase expression in MPNST tumor biopsies. Note that EGFR and cErbB expression is lower than in the xenograft model (Figure 1), although proliferation rate (Ki67 staining) is comparable. PDGFR expression is comparable to that observed in the xenograft model. These data indicate that the expression profile remains stable in tumor xenografts, but relative and absolute expression levels vary. In addition, regulation of MPNST tumor growth in NF1 patients may be less dependent on the EGF pathway than in the xenograft model.

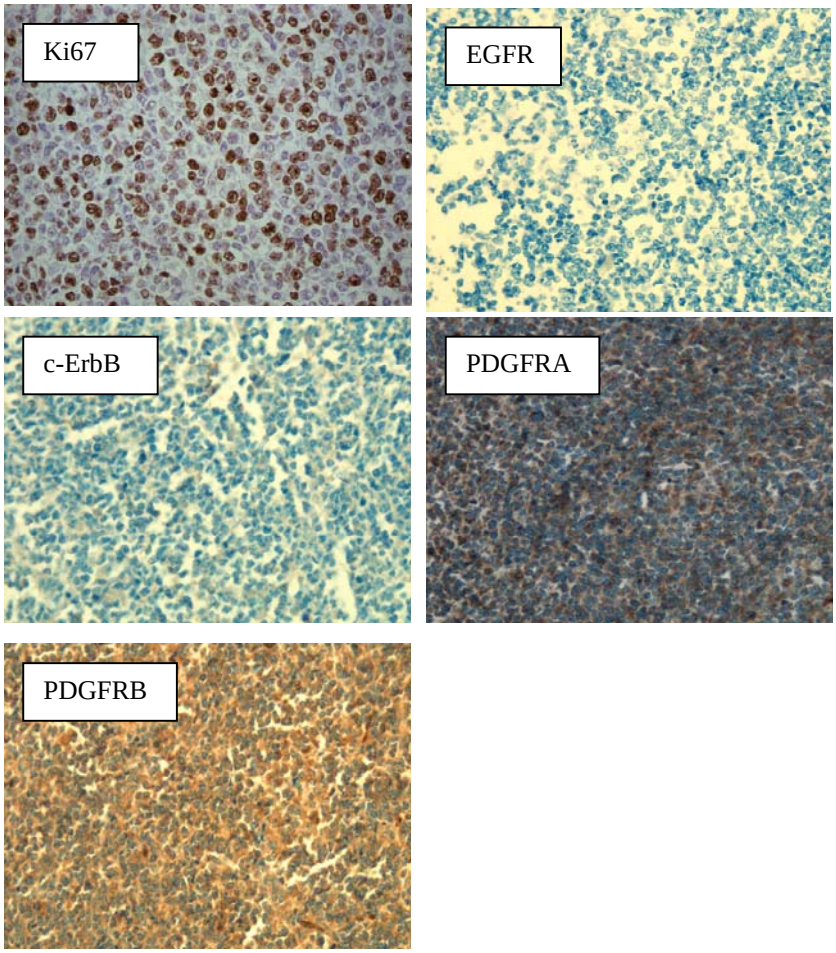


Figure 3: Tumor growth rate of MPNST cell xenograft in mice. Tumors start exponential growth after a prolonged incubation time. $5x10^6$ cells were injected subcutaneously. Shown is cell line S462.

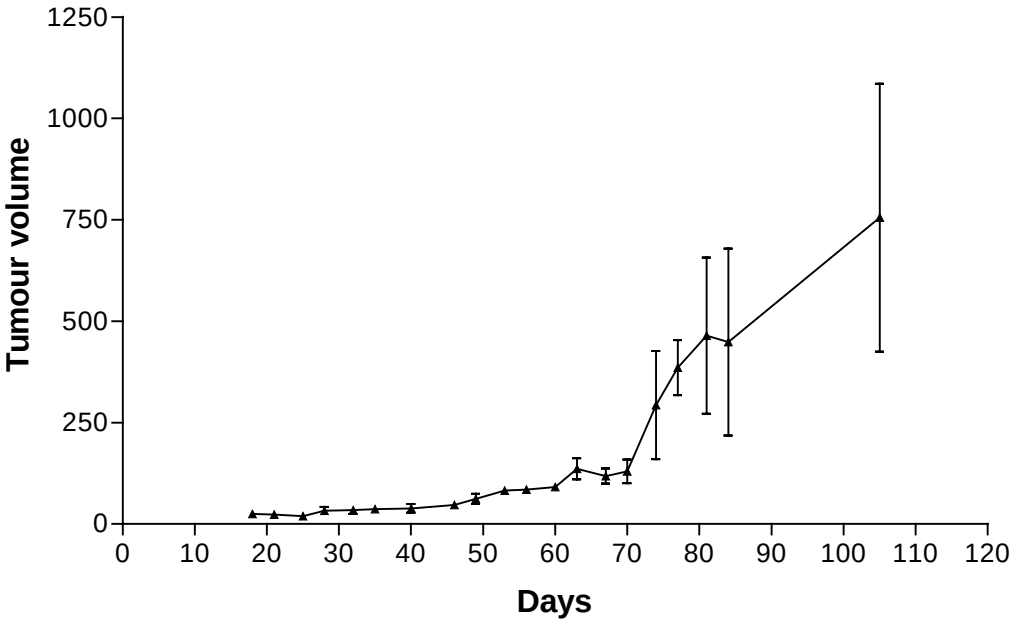
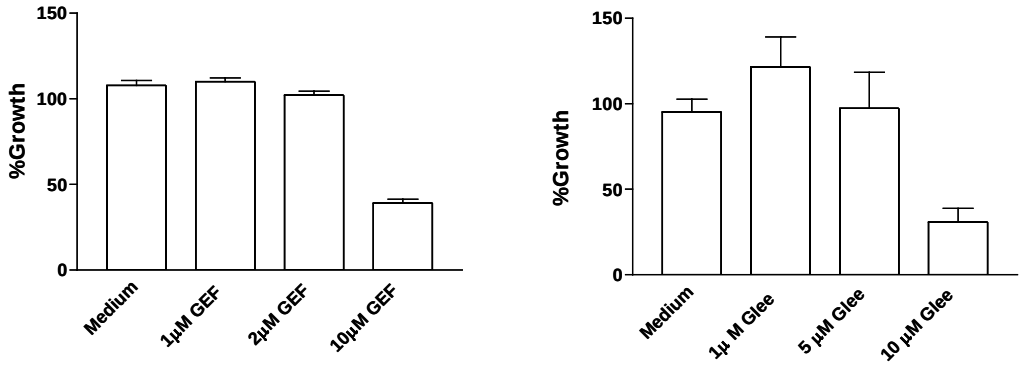


Figure 4: Effects of Gleevec (Glee, inhibits PDGFR and Kit) and Gefitinib (GEF, inhibits EGFR) on proliferation of the MPNST line S462. (5-BrDU incorporation). Both, Gleevec and Gefitinib show a significant effect on S462 cells in vitro, but only at the highest drug concentration.



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Mutation and expression of *PDGFRA* and *KIT* in malignant peripheral nerve sheath tumors, and its implications for imatinib sensitivity

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Platelet-derived growth factor receptor alpha (PDGFRα) and c-Kit are receptor tyrosine kinases. Both are targets of the tyrosine kinase inhibitor imatinib mesylate which is approved for treatment of some cancers. In order to assess the role of PDGFRα and c-Kit in malignant peripheral nerve sheath tumours (MPNST) we examined human tumours for structural alterations, protein and ligand expression. We investigated 34 MPNST, 6 corresponding plexiform neurofibromas (pNF) and 1 MPNST cell culture from 31 patients for mutations and polymorphisms in *PDGFRA* (exon 2–21) and *KIT* (exon 9, 11, 13, 17). *PDGFRA* was amplified in seven tumours from six patients and MPNST cell culture S462. *KIT* was amplified in five tumours from four patients and in the cell culture. Two MPNST carried somatic *PDGFRA* mutations in exons coding for the extracellular domain. In addition we detected several polymorphisms in *PDGFRA*. No point mutations or polymorphisms were detected in the four *KIT* exons analysed. PDGFRα expression was present in 21 of 28 MPNST patients (75%) and the MPNST cell culture. Expression analysis of PDGFRα ligands in MPNST and neurofibromas revealed that PDGF-A was more widely expressed than PDGF-B. Focal c-Kit expression was detected in 2 of 29 (7%) MPNST patients. Imatinib treatment of MPNST cell culture S462 exerted a growth inhibitory effect and prevented PDGF-AA induced PDGFRα phosphorylation. In summary, *PDGFRA*, *PDGF* and *KIT* dysregulation as well as growth inhibition of cell culture S462 by imatinib may suggest that MPNST patients benefit from treatment with imatinib.

Introduction

Malignant peripheral nerve sheath tumours (MPNST) are very aggressive tumours with poor prognosis. Approximately half of the MPNST occur in the setting of Neurofibromatosis type 1 (NF1), a hereditary tumour syndrome with an incidence of 1:3500 (1). MPNST in NF1 patients is the major cause for reduced life expectancy with only 21% of NF1 patients surviving longer than 5 years after diagnosis of MPNST (2).

The *NF1* gene on chromosome 17q11.2 encodes the large protein neurofibromin, which acts as negative regulator of ras. Functional neurofibromin is lost in NF1 associated nerve sheath tumours. However, not much is known about additional molecular aberrations underlying transformation or malignant progression of nerve sheath tumours. However, *TP53* and *CDKN2A* have been shown to harbour mutations in a subgroup of MPNST (3–5). In addition, gene amplification and increased transcription of *EGFR* have been detected in MPNST (6,7). MPNST in NF1 patients frequently arise from malignant progression of plexiform neurofibromas (pNF). Dermal neurofibromas (dNF), which develop in nearly all NF1 patients, virtually have no risk of malignant transformation. With the exception of *NF1* loss, little is known about genetic aberrations in neurofibromas.

We have recently demonstrated higher expression of platelet-derived growth factor receptor alpha (PDGFRα) in MPNST than in benign nerve sheath tumours (8). Because ligands of PDGFRα are powerful mitogens for Schwann cells (9), the PDGF system might contribute to malignant progression of nerve sheath tumours. The PDGF system is complex with two receptor genes forming three receptor types (PDGFRα and PDGFRβ homodimers and PDGFRαβ heterodimers). These receptors may bind ligands encoded by four genes building at least five different dimers (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD) with different receptor-binding specificities (10). Expression of c-Kit and its ligand stem cell factor was found in MPNST cell cultures (11,12) and c-Kit expression was documented in an MPNST (13).

PDGFRα and c-Kit belong to the type III subfamily of receptor tyrosine kinases. Both have been reported to be strongly expressed and/or mutated in gastrointestinal stromal tumours (GIST) and gliomas (14,15).

PDGFRA and *KIT* have attracted special attention since it became evident that their protein products were among the tyrosine kinases inhibited by imatinib mesylate (Glivec®, STI571). Imatinib treatment yields high response rates in patients with GIST and chronic myeloid leukemia (CML) (16,17). The major target of imatinib in GIST is mutant c-Kit, whereas the inhibited kinase in CML is the Bcr–Abl fusion protein. Successful treatment with imatinib has also been reported for patients with dermatofibrosarcoma protuberans. This disease is characterized by a chromosomal translocation leading to overexpression of PDGF-B, which is bound by all 3 PDGF receptor types (18).

Our previous findings of increased PDGFRα levels in MPNST led us to examine MPNST for activating mutations or amplifications. Activation of PDGFRα and c-Kit would

Abbreviations: DMSO, dimethylsulfoxide; GIST, gastrointestinal stromal tumours; MPNST, malignant peripheral nerve sheath tumours; NF1, neurofibromatosis type 1; PDGFRα, platelet-derived growth factor receptor alpha; pNF, plexiform neurofibromas.

Table I. Primer sequences and product size

	Forward	Reverse	Product size in bp
DNA primer			
KIT exon 9a	TTTCCTAGAGTAAGCCAGGGC	GTTGTAAGCCTTACATTCAACCG	180
KIT exon 9b	AGTGCATTCAAGCACAATGG	GACAGAGCCTAAACATCCCC	146
KIT exon 11	CTATTTTTCCCTTTCTCCCC	TACCCAAAAAGGTGACATGG	193
KIT exon 13	TTTGCCAGTTGTGCTTTTTG	CAGCTTGGACACGGCTTTAC	176
KIT exon 17	TGGTTTTCTTTTCTCCTCCAA	TGCAGGACTGTCAAGCAGAG	185
PDGFRA exon 2	TCCAGGGTTGTTTCTATTTGC	GACACCCAAACAAGGAACTCA	157
PDGFRA exon 3/1	CCTATTCAGAGCGTGCTTCC	AGGCCGCTGTTGTTTTCTT	218
PDGFRA exon 3/2	GGGGAGAGTGAAGTGAGCTG	CCAACTCACCTGGCACATAG	226
PDGFRA exon 4/1	TCTGGATTTATGTGTAAAGGTGAAA	TCCCATTAAAGCCCTGTCTG	239
PDGFRA exon 4/2	TCCCGAGACTCCTGTAACCTT	CACGCACCTTATGATTTTGC	229
PDGFRA exon 5	TGTGGATTTTTAGGCCCTTG	CATTGCACGTTTTGAGGGTA	195
PDGFRA exon 6	GGTTTTCTTCCCCTTTTGCT	GCAGCATGGACAACTGACAT	235
PDGFRA exon 7	CTCGGGATCCATATGTGGTAA	CGCCTCTGATGCACACTAAA	295
PDGFRA exon 8	TGCTTGTTGAAACAAAATCCTTT	CACTCATCTACAGAGCTAGCATTATC	194
PDGFRA exon 9	TCTGGGACACGAGCTATTCC	CTGACCACGAAAGAAGAAGACA	212
PDGFRA exon 10	GGCCCTATACTTAGGCCCTTT	TCCTGACTGTTGAGGAACTCAC	247
PDGFRA exon 11	GCATGTCTGCCAGGAAACTT	TGCTTGTTCTCATTGGCTTC	197
PDGFRA exon 12	TCCAGTCACTGTGCTGCTTC	GCAAGGGAAAAGGGAGTCTT	261
PDGFRA exon 13	CGTCTGGAGTTTTTGGGTGT	CCCCAGGAAGGAGCACTTAC	167
PDGFRA exon 14	GCTCAGCTGGACTGATATGTGA	CCAGTGAAAATCCTCACTCCA	185
PDGFRA exon 15	ACCCATCTCCTAACGGCTTT	CAGCAACATCTCTCTTTGCAC	210
PDGFRA exon 16	GGCACCCTGGGTAAGATTTC	CACTGGAAAGTCCCAACACC	248
PDGFRA exon 17	CCTGCCAGCACCAATACAT	GGGTCTAAAAAGGTCTGTGTTCC	186
PDGFRA exon 18	ACCATGGATCAGCCAGTCTT	TGAAGGAGGATGAGCCTGACC	252
PDGFRA exon 19	AACTGTCTCCCTCCTTCCTTG	GCCCAAATAAGCAGCAATGT	167
PDGFRA exon 20	TGGTGTTTTATTGTTTGGCTTTT	CCCCCTAGACCCACAGACT	180
PDGFRA exon 21	TCTTGAGTTCTGTCCCCACA	CACCCACAGATCCAAACACA	148
Desmin exon 8	ACTCCCAGCCCCTGGTATAG	AGGGTAAGGAGCCCAGACAG	180
cDNA primer			
PDGFRA fragment 1	GACTTCCCATCCGGCGTTC	TTGACCTCCCTGGTAGCCT	894
PDGFRA fragment 2	TGGAGATTACGAATGTGCTG	CCAAGCACTAGTCCATCTCT	933
PDGFRA fragment 3	CTTATGACTCAAGATGGGAG	CAGACATCACTCAGTGTGGT	954
PDGFRA fragment 4	CTCCTGAGAGCATCTTTGAC	AAGTGGAAGGAACCCCTCGA	712
PDGFA	ACACGAGCAGTGTCAAGTGC	GGCTCATCCTCACCTCACAT	200
PDGFB	GGCATGCAAGTGTGAGACAG	GTCTTGTCATGCGTGTGCTT	171
RPS3	CTGGGCATCAAGGTGAAGAT	AGACCCTGTTATGCTGTGGG	205

render MPNST attractive candidates for imatinib treatment. Since there are only limited treatment options for MPNST patients new treatment possibilities are required.

Materials and methods

Tumour tissue, DNA and RNA extraction

Tumour samples were collected from University Hospital Eppendorf (Hamburg, Germany), Robert-Rössle-Hospital (Berlin, Germany), Otto-von-Guericke-University (Magdeburg, Germany) and Charité University Hospital (Berlin, Germany). The study contained 40 nerve sheath tumours (34 MPNST and 6 pNF) from 31 patients and MPNST cell culture S462, which was established from MPNST 24472 (19). Twenty-six patients were diagnosed with NF1. Following initial diagnosis in local neuropathologies, all tumour samples were reviewed by the same pathologist (A.F.O.). Histopathological examination was based on the modified FNCLCC system (20). Eleven tumour samples have already been analysed for gene expression profiles (6). Before extraction of DNA, RNA and protein tumour samples were examined by histology to exclude contaminating non-tumourous portions. In case of frozen material DNA and RNA was extracted using TRIzol reagent from Invitrogen (Karlsruhe, Germany). DNA extraction from paraffin embedded material was carried out according to the QIAamp DNA Mini Kit protocol (Qiagen, Hilden, Germany). Four MPNST (24626, 24772, 24776 and 24324) contained adjacent pNF tissue. DNA from pNF and MPNST areas were separately extracted. A c-Kit positive GIST was kindly provided by Prof. Gottschalk (Hamburg) and served as positive control for immunohistochemistry. Microdissection of skeletal muscle and vascular endothelial cells was performed with two cases (24748 and 24772) because blood was not available. The PALM Laser Microbeam System (Bernried, Germany) was employed to dissect ~2000 cells from paraffin sections stained with toluidine blue as described previously (21). The investigations were carried out with the informed consent of the patients.

SSCP and sequencing

Electrophoresis of PCR products of *PDGFRA* exon 2–21 and *KIT* exon 9, 11, 13, 17 was performed on polyacrylamid gels applying 500 V and 6 mA for 18 h. All PCR products showing a mobility shift were confirmed by an independent PCR and compared with PCR products of corresponding normal tissue (blood or microdissected normal cells). Aberrantly migrating SSCP bands were excised and the DNA was extracted. After reamplification, PCR products were sequenced bidirectionally on a semiautomated sequencer (model 377; Applied Biosystems, Foster City, CA). Sequences were compared to *PDGFRA* NM_006206 and *KIT* X06182. Primer sequences are compiled in Table I. Amplification and gel conditions are available on request.

Gene amplification analysis by real-time PCR

Quantitative real-time PCR was performed with SYBR green I (1:5000 dilution, Molecular Probes, The Netherlands) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR products of the target genes *KIT* (Exon 17, PCR product of 185 bp) and *PDGFRA* (Exon 21, PCR product of 148 bp) were compared to the reference gene *DES* (Exon 8, PCR product of 180 bp) on chromosome 2q35 encoding desmin. This region appears not to have gains of chromosomal material in MPNST (22). Primer sequences are given in Table I. PCR efficiency, determined by serial dilution of DNA, demonstrated similar results for target and reference genes. All samples were analysed in duplicate in 25 μl reaction mixes containing 1.25 U of Platinum *Taq* DNA Polymerase (Invitrogen). Amplification conditions are available on request. Evaluation of data were performed using the ΔΔCt method: $\Delta\Delta Ct = \Delta Ct$ tumour DNA $- \Delta Ct$ blood DNA. ΔCt (threshold cycles) is the Ct of the reference gene minus the Ct of the target gene. Fold increase of the target genes (*PDGFRA* and *KIT*) was calculated by $2^{(\Delta\Delta Ct)}$ and values of ≥ 1.7 were defined

as gene amplification. Tumours conspicuous for gene amplification were verified in an independent PCR run. DNAs from glioblastomas with known *PDGFRA* amplification served as positive controls (15).

RT–PCR analysis

RT of 2 μg DNA free RNA was achieved with the SuperScript™ First-Strand Synthesis System (Invitrogen). PCR were performed in a volume of 20 μl containing cDNA transcripts equivalent to 45 ng RNA. For the detection of large deletions in *PDGFRA* 10 MPNST (21852, 24784, 26580, 26582, 26584, 26586, 26588, 26592, 24472, 21914) and the cell culture S462 were analysed using four overlapping primer pairs covering the whole coding region of *PDGFRA*. Primer sequences are given in Table I. PCR fragments were separated on 1.5% agarose gels allowing size differences of 50 bp to be visualized. For semi-quantitative ligand determination 35 PCR cycles were performed for *PDGFA* and *PDGFB*, and 32 cycles were performed for the reference gene *RPS3*. Amplification conditions are available on request.

Immunohistochemistry and scoring

Immunohistochemistry was performed with a Ventana Benchmark™ automate (Ventana, Strasbourg, France). The antibodies against PDGFRα (C-20, dilution 1:100) and PDGF-A (N-30, dilution 1:50) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The c-Kit antibody (A4502, dilution 1:400) was obtained from Dako (Hamburg, Germany). Antigen retrieval was enhanced by heating. Primary antibodies were incubated for 30 min at 40°C. Negative controls without primary antibodies were carried out and did not produce signals. Scoring was performed according to the percentage of positive cells: <5% was classified as negative (−), 6–100% was classified as positive. Positive cells (6–30%) were scored with +, 31–60% with ++, >60% with +++. A repeated test (blinded) gave similar results.

Immunocytochemistry

MPNST cells (2×10^4) per well were seeded on Permanox chamberslides (Nunc, Wiesbaden, Germany). Cells were fixed with acetone the following day. The same antibodies used for immunohistochemistry were applied in a 1:50 dilution. Incubation of primary antibodies was performed for 2 h at room temperature. Visualization was performed with Cy3- (Dianova, Hamburg, Germany) or Alexa488- (Invitrogen) conjugated anti-rabbit antibodies (dilution 1:100). Negative controls without primary antibodies were carried out and did not produce signals.

Western blot

Tumour lysates were heat denaturated and loaded on to 7.5% acrylamide gels for subsequent protein separation. After transfer of proteins to nitrocellulose membranes, the membranes were blocked in 3% non-fat dry milk with 0.05% Tween-TBS for 1 h and incubated overnight at 4°C with anti-PDGFRα antibody (C-20, dilution 1:200). After washing, the membranes were incubated for 1 h with biotin-conjugated second antibodies, washed and incubated 1 h with ExtrAvidin from Sigma (dilution 1:2000). Visualization was performed with ECL (Amersham Biosciences, Freiburg, Germany). Lysates were adjusted to β-actin expression levels which were determined with the anti-β-actin antibody AC-15 (dilution 1:6.000) from Sigma (Munich, Germany). Phosphorylation was detected with anti-p-Tyr (PY99, dilution 1:10.000) from Santa Cruz Biotechnology.

Cell culture assays

During imatinib inhibition the MPNST cell culture S462 (<18 passages) was maintained in DMEM Glutamax-I with 5% FBS from Invitrogen (Karlsruhe, Germany). Imatinib mesylate was kindly provided by Novartis Pharma AG (Basel, Switzerland) and dissolved in dimethyl sulphoxide (DMSO). Cells (2×10^3) were seeded in 300 μl medium into 24 well plates and were allowed to adhere. Imatinib was added in 100 μl to final concentrations of 2 μM and 10 μM with no more than 0.1% DMSO. Negative controls contained 0.1% DMSO only. The 300 μl of medium containing respective imatinib or DMSO concentrations were exchanged on day 3 and 5. Cell proliferation was evaluated on day 4 and 7 post-imatinib treatment with the CellTiter 96 AQueous One Solution Cell Proliferation Assay from Promega (Mannheim, Germany) by measurement of absorbance at 490 nm. The experiments were performed in duplicate and repeated thrice with comparable results.

Imatinib effect on PDGFRα phosphorylation was determined in 6 well plates in duplicates. Cells (5×10^5) were seeded per well in DMEM with 10% FBS. The next day medium was changed to serum-free DMEM. After 24 h imatinib or DMSO was added and cells were incubated for 30 min. Cells were then stimulated with PDGF-AA (50 ng/ml) for 10 min, washed with phosphate-buffered saline (PBS), scraped, centrifuged and resuspended in lysis buffer [1% Triton X-100, 100 mM NaCl, 50 mM Tris–HCl (pH 7.5) and 5 mM EDTA]. Protease inhibitor cocktail (Roche, Penzberg, Germany) and phosphatase inhibitor cocktail 2 (Sigma, Saint Louis, Missouri) were added.

Stimulation with PDGF-AA and PDGF-BB (Oncogene, San Diego, CA) was performed in 6 well plates with 50 ng PDGF per ml serum-free DMEM for 48 h. Each well contained 10^5 cells seeded in DMEM with 10% FBS. The next day medium was switched to serum-free DMEM. Cell line ST88-14 and DBTRG (kindly provided by A. Kurtz and E. Elstner) were cultured in DMEM Glutamax-I with 10% FBS.

Results

Mutations and polymorphisms

We investigated 34 MPNST, 6 pNF and 1 MPNST cell culture from 31 patients for *PDGFRA* mutations (exon 2–21) and *KIT* mutations (exon 9, 11, 13, 17). Because *KIT* mutations in GIST mostly occur in exon 9 and 11 but also in exon 13 and 17 we restricted our analysis to these four exons (23). We detected somatic *PDGFRA* mutations in 2 MPNST (Figure 1A and B). MPNST 24748 had a mutation in exon 4 (CCT>TCT, codon 130) leading to the non-conservative amino acid exchange from proline to serine. MPNST 24740 harboured a somatic mutation in exon 10 (GTC>GCC, codon 469) leading to an exchange from valine to alanine.

Because a transforming PDGFRα deletion mutant (loss of exon 8 and 9) was reported in a glioblastoma (24) we examined *PDGFRA* cDNA in order to detect large deletions that would not be recognized by SSCP. Analysis of 10 MPNST and cell culture S462 did not reveal large deletions (data not shown).

We found six different single nucleotide polymorphisms in *PDGFRA* which are listed in Table II. Three of 31 MPNST from individual patients were heterozygous for the 478Pro allel and exhibited allel frequencies of $f(Ser^{478}) = 0.950$ and $f(Pro^{478}) = 0.050$. To determine the allel frequency in the general population we investigated 150 blood samples. The allel frequency was $f(Ser^{478}) = 0.873$ and $f(Pro^{478}) = 0.127$. Individuals with the 478Pro variant in exon 10 also carried the silent polymorphism in exon 7. Recently, we detected the 478Pro polymorphism in 14 of 103 gliomas (13 heterozygous and 1 homozygous) (25). The allel frequency in glioma was $f(Ser^{478}) = 0.927$ and $f(Pro^{478}) = 0.073$.

The pNF 28572 and corresponding normal cells carried an allelic variant lacking 18 bp in exon 7. The 18 bp deletion variant seen in a single patient has not been reported before and we did not find it in 150 control individuals (data not shown). The four *KIT* exons analysed did not harbour mutations or polymorphisms.

Gene amplification of PDGFRA and KIT

PDGFRA and *KIT* amplification was investigated by real-time PCR. Eight samples (seven solid tumours and cell culture S462) from six patients showed *PDGFRA* amplification by a factor of 1.8 or more relative to the normal gene dose. *KIT* amplifications were detected in six samples (five tumours and cell culture S462) from four patients. Five samples (24472, 24784, 21914, 22318, cell culture S462) showed amplification of both, *PDGFRA* and *KIT*. Gene amplification results are summarized in Table III.

Expression of receptors PDGFRα and c-Kit

PDGFRα expression was determined in 32 MPNST from 28 patients. Immunoreactivity was observed on sections from 21 of 28 MPNST patients (75%). Twelve MPNST (38%) expressed PDGFRα in >60% of tumour cells, 7 MPNST (22%) in 31–60% of the tumour cells and 4 MPNST (13%) in 6–30% of the tumour cells. Five pNF were also evaluated

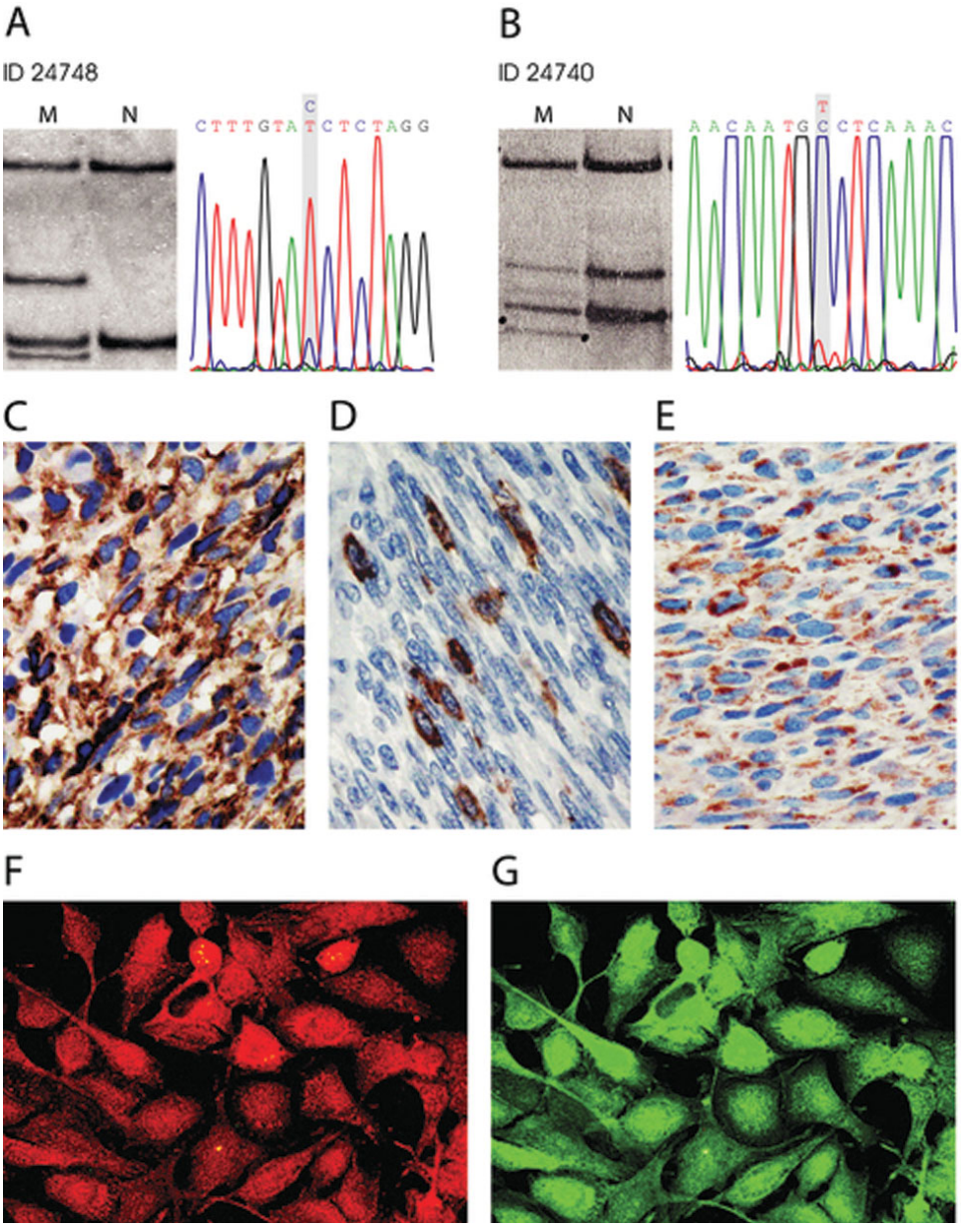

Fig. 1. Mutation and expression in MPNST. (**A**) and (**B**) SSCP gels and sequences of the shifted *PDGFRA* bands. MPNST 24748 shows a T to the wild-type C exchange and MPNST 24740 a C to the wild-type T exchange. (C–E) Immunohistochemistry. PDGFRα expression in MPNST 21852 (**C**), c-Kit expression in MPNST 24694 (**D**), PDGF-A expression in MPNST 24748 (**E**). (**F**) and (**G**) shows cell culture S462 double stained with antibodies to PDGF-A red, (F) and PDGFRα green, (G). Original magnification: 400×.

and 4 showed PDGFRα expression. Three of these pNF localized adjacent to MPNST and 2 of them showed lower PDGFRα expression than the MPNST areas (MPNST 24626/pNF 28578 and MPNST 24772/pNF 28572). MPNST 24324 and corresponding pNF 28580 contained >60% PDGFRα positive tumour cells in both parts of the tumour. An example of immunohistochemistry is shown in Figure 1C. Results are compiled in Table III. Examination of 5 MPNST and cell culture S462 for PDGFRα expression by western blot revealed bands at the expected size of 185 kDa in four samples (Figure 2). Further 6 neurofibromas analysed by western blotting showed little or no PDGFRα signals (data not shown). The western blot results were in accordance with immunohistochemistry. All tumour samples positive for PDGFRα in western blot (21914, 21852, 24472 and corresponding cell culture S462) were also positive in immunohisto- and cytochemistry (Figure 1C and G). No signals were detected in MPNST 24480 and 24784. Accordingly, these tumours were negative or showed PDGFRα expression in only a minority of cells. With the exception of pNF 28578, all tumours with *PDGFRA* amplification expressed PDGFRα.

Immunohistochemistry revealed focal c-Kit expression in 2 MPNST (Figure 1D). C-Kit positive mast cells within the tumours and a c-Kit expressing GIST served as controls.

Expression of growth factors PDGFA and PDGFB

In order to examine PDGFRα ligand expression in neurofibromas and MPNST we performed RT–PCR for *PDGFA* and *PDGFB* in 5 dNF, 3 pNF, 8 MPNST and 2 MPNST cell cultures and the glioblastoma cell line DBTRG. *PDGFA* was expressed in most tumours whereas *PDGFB* was detected in only 1 pNF but the majority of MPNST (Figure 3).

PDGF-A expression was determined in 30 MPNST from 26 patients. Immunoreactivity was observed on sections from 21 of 26 MPNST patients (81%). Fifteen MPNST (50%) expressed PDGF-A in >60% of tumour cells, 3 MPNST (10%) in 31–60% of the tumour cells and 5 MPNST (17%) in 6–30% of the tumour cells. Five pNF were also evaluated and 4 showed PDGF-A expression. Immunocytochemistry revealed strong PDGF-A expression in MPNST cell culture S462 (Figure 1F). Results for individual tumours are given in Table III.

Table II. Allelic variants of *PDGFRA* in MPNST

Exon	Codon	Triplett	Amino acid	Patient ID
3	79	GGC>GAC	G>N	26592
7	Del 348–353	Del 18 bp	Deletion of SWLKNN	24772
7	313	GGT>GGG	Silent	24308, 24624/24626, 26586, 27724
10	478	TCC>CCC	S>P	24308, 24624/ 24626, 26586
13	603	GCG>GCA	Silent	26586
16	764	CGT>CAT	R>H	24740
18	824	GTC>GTT	Silent	24308, 26586, 26588

Inhibition of MPNST cell culture proliferation and phosphorylation of PDGFRα by imatinib

The inhibitory effect of imatinib was tested with concentrations of 2 and 10 μM. Use of 2 μM imatinib led to an 11% reduction in proliferation on day 4 and a reduction of 39% on day 7 as compared to untreated control cells. The effect of 10 μM imatinib was more pronounced with 58% inhibition on day 4 and 66% on day 7 post-exposure (Figure 4A). This corresponds to a biologic IC_{50} of <10 μM. Next, we evaluated if concentrations of 2 μM and 10 μM imatinib would inhibit ligand induced phosphorylation of PDGFRα. In fact, both imatinib concentrations prevented PDGF-AA induced phosphorylation in S462 cells which corresponds to a pharmacologic IC_{50} <2 μM (Figure 4B).

Stimulation with PDGF-AA and PDGF-BB increased proliferation of S462 cells under serum-free conditions by a

Table III. Mutation and protein expression of *KIT* and *PDGFRA* in peripheral nerve sheath tumors

No.	Patient ID	Tumour entity	Grade	NF1	PDGFRA mutation	PDGFRα IHC	PDGFRα WB	PDGF-A IHC	KIT mutation	c-Kit IHC
1	24256¥	MPNST	3	Yes	–	+++	nd	++	–	–
2	24740¥	MPNST	3	Yes	Codon 469 GTC>GCC	+	nd	+++	–	–
3	24304	MPNST	1	Yes	–	nd	nd	nd	–	–
4	24326	MPNST	2	Yes	–	++	nd	+++	–	–
5	24624*	pNF	1	Yes	–	++	nd	+	–	–
6	24626*	MPNST	2	Yes	2.8amp	+++	nd	+++	–	–
7	28578*	pNF	1	Yes	2.4amp	–	nd	++	–	–
8	24534	MPNST	3	Yes	–	–	nd	+++	–	–
9	24668	MPNST	3	Yes	–	++	nd	+	–	–
10	24670	MPNST	3	Yes	–	+++	nd	+	–	–
11	24748	MPNST	3	Yes	Codon 130 CCT>TCT	++	nd	+++	–	–
12	24772+	MPNST	2	Yes	–	+++	nd	+	–	+ focal
13	28572+	pNF	1	Yes	–	+	nd	–	–	–
14	24776∼	MPNST	1	Yes	–	+++	nd	+	–	–
15	28576∼	pNF	1	Yes	–	nd	nd	nd	–	nd
16	24472#	MPNST	3	Yes	10.3amp	++	+	+++	5.5amp	–
17	S462#	Cell line	–	Yes	5.1amp	+++	+	+++	7.9amp	–
18	24480	MPNST	2	Yes	–	–	–	nd	–	–
19	24484	MPNST	3	Yes	3.7amp	nd	nd	nd	–	nd
20	24476	MPNST	2	Yes	–	–	nd	+++	–	–
21	22476$	pNF	1	Yes	–	+	nd	+++	9.3amp	–
22	24784$	MPNST	1	Yes	2.7amp	+	–	+++	9.6amp	–
23	21914	MPNST	2	Yes	5.9amp	++	+	+	1.8amp	–
24	21852§	MPNST	2	Yes	–	+++	+	+++	–	–
25	22318§	MPNST	3	Yes	2.0amp	+	nd	–	3.3amp	–
26	24308	MPNST	3	Yes	–	+	nd	+++	–	–
27	24310	MPNST	2	Yes	–	–	nd	nd	–	–
28	24324%	MPNST	1	Yes	–	+++	nd	+++	–	–
29	28580%	pNF	1	Yes	–	+++	nd	+++	–	–
30	24332	MPNST	2	Yes	–	+	nd	++	–	–
31	24354$	MPNST	1	Yes	–	++	nd	+++	–	–
32	24694	MPNST	2	Yes	–	–	nd	–	–	+ focal
33	26592	MPNST	2	Yes	–	+++	nd	–	–	–
34	28650	MPNST	2	Yes	–	++	nd	++	–	–
35	28652	MPNST	1	Yes	–	–	nd	+++	–	–
36	27724	MPNST	3	Yes	–	–	nd	+++	–	–
37	26580	MPNST	3	No	–	nd	nd	nd	–	nd
38	26582	MPNST	3	No	–	+++	nd	–	–	–
39	26584	MPNST	2	No	–	+++	nd	+++	–	–
40	26586	MPNST	2	No	–	+++	nd	–	–	–
41	26588	MPNST	3	No	–	+++	nd	–	–	–

Amplification status (amp) is indicated by fold increase relative to the normal gene dose. ID: tumour identification number. NF1: NF1 status of the tumour patient. IHC: immunohistochemistry. WB: western blot. symbols ¥, $, %, #, §, *, + indicate tumours belonging to the same patient. nd: not determined (lack of material). Tumours were graded according to the modified FNCLCC system.

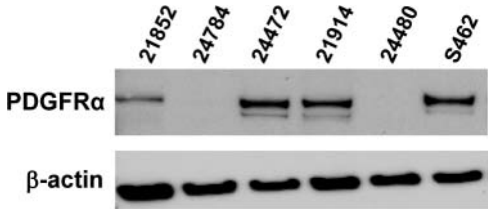

Fig. 2. Western blot of primary MPNST and MPNST cell culture S462 with antibodies to PDGFRα and β-actin.

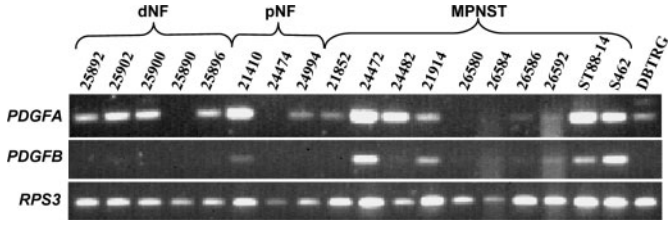

Fig. 3. Expression of *PDGFA* and *PDGFB* in dermal neurofibromas (dNF), plexiform neurofibromas (pNF), MPNST, MPNST cell lines and the glioblastoma cell line DBTRG. *RPS3* served as reference gene.

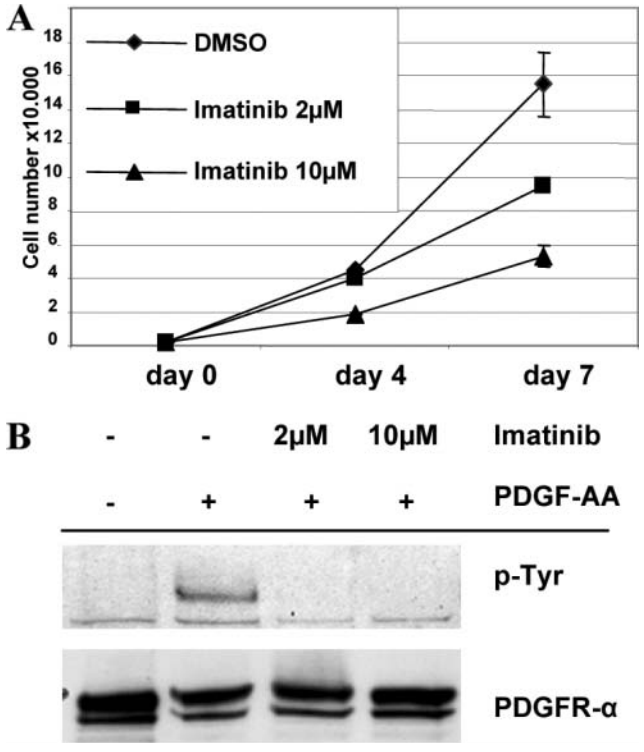

Fig. 4. (**A**) Effect of imatinib on proliferation of MPNST cell culture S462. (**B**) Inhibition of PDGF-AA induced phosphorylation of PDGFRα by 2 and 10 μM imatinib. Lysates of treated cells were resolved by electrophoresis and transferred to membranes. Membranes were probed with the indicated antibodies.

factor of four in comparison to the untreated controls (data not shown).

Discussion

This study is the first to demonstrate molecular aberrations of receptor tyrosine kinase genes *PDGFRA* and *KIT* in MPNST. We detected gene amplification of *PDGFRA* in 6 and somatic point mutations in 2 of 31 patients with MPNST. Structural alterations of *PDGFRA* were therefore present in 8 of 31 (26%) patients with MPNST. The 2 point mutations localized to exon 4 and exon 10 of *PDGFRA*, which encode parts of the extracellular domain of PDGFRα. *PDGFRA* exon 10 corresponds to *KIT* exon 9 which is the second most mutated *KIT* exon in GIST (23). We found valine substituted by alanine in codon 469. This valine, located in the Ig-like domain of the receptors, is conserved in c-Kit and corresponds there to codon 459. Mutations in the extracellular domain may modulate ligand-binding and dimerization, thereby indirectly influencing tyrosine kinase activity. Finally, mutations of *PDGFRA* in MPNST occur as frequent as *EGFR* amplifications, which have also been found in 26% of MPNST (7). Other frequent alterations in MPNST include *NF1*, *CDKN2A* and *TP53* deletions and/or mutations (5,7,19,26). Until today not many tumour entities with mutations in *PDGFRA* have been described. Recently it was shown, that a subset of about 35% of GISTs lacking *KIT* mutations carried mutations in *PDGFRA* (27,28) indicating that either *KIT* or *PDGFRA* aberrations contribute to the development of these tumours.

We detected 7 allelic variants of *PDGFRA* present in both, tumour and reference tissues. Three of them led to an amino acid exchange and one to a truncated protein. The 478Pro variant has been described before and functional analysis revealed no constitutive phosphorylation like 2 PDGFRα gain-of-function mutants (25,28). We found a variant of *PDGFRA* with an 18 bp deletion resulting in a truncated protein lacking the 6 amino acids SWLKNN in a single patient (Table II). This deletion is located in the extracellular Ig-like domain IV and may, therefore, modulate ligand-binding. Protein extracts for western blotting were available from 5 MPNST patients. Three samples showed expression of PDGFRα, 2 of which were detected with amplification. Two samples with weak PDGFRα expression were derived from one patient with, and one without, gene amplification. Immunohistochemistry demonstrated PDGFRα expression in the majority of MPNST patients (75%). A cell culture established from MPNST 24472 exhibited similar features regarding *PDGFRA* amplification and PDGFRα expression as the native tumour. Gene amplification of *KIT* was detected in 4 of 31 patients. No point mutations were observed. Immunohistochemistry revealed c-Kit expression in 2 of 29 patients (7%). Both patients with focal c-Kit expression did not harbour *KIT* amplifications. Our data demonstrating limited c-Kit expression in MPNST are in accordance with a recent study that found weak c-Kit expression in 1 of 18 MPNST (29).

With the exception of pNF 22476, all tumours with *KIT* amplification also exhibited *PDGFRA* amplification. Notably, *PDGFRA* and *KIT* map next to each other with a distance of 359 kb on the chromosomal segment 4q12. Thus, the amplicon in the majority of the MPNST in our series contains more than one gene. Amplicons containing several genes have been described previously such as the frequent amplicons on chromosomal segment 12q13–q15 in glioblastoma containing *MDM2*, *SAS*, *CDK4* and in some cases also *GADD153*, *GLI*, *RAP1B*, *A2MR* and *IFNG* (30). In MPNST the amplification of both *PDGFRA* and *KIT* may have profound effects on signal transduction. However, our observation of higher frequency of *PDGFRA* aberrations and stronger expression of PDGFRα may suggest that this gene provides a major selective advantage for MPNST tumour cells and that *KIT*, due to infrequent

expression of the protein, may be co-amplified as an 'innocent bystander'.

Because point mutations in *PDGFRA* appear to be infrequent in MPNST we suggest that an autocrine loop of the PDGF system supports proliferation and angiogenesis. We could show, that expression of PDGFR ligands PDGF-A and PDGF-B is common to nerve sheath tumours. *PDGFA* was more widely expressed than *PDGFB* especially in neurofibromas. Similar mechanisms are known to play a role in gliomas (31,32). In addition to an autocrine loop, paracrine stimulation of the PDGF system may also contribute to tumour proliferation because fibroblasts are known to be a source for PDGF. There is evidence for a direct link between NF1 and PDGF. It was shown that Schwann cells derived from Nf1−/− mice expressed *PDGFB* which was absent in Nf1+/+ mice (33).

Patients with GIST respond to treatment with imatinib, an inhibitor of the protein tyrosin kinase c-Kit (16). Imatinib does not selectively inhibit c-Kit but also interferes with other receptor tyrosine kinases such as PDGFRα (34,35). We therefore analysed growth of MPNST cells at imatinib concentrations of 2 and 10 μM employing cell culture S462, which was shown to harbour loss of heterozygosity in genes encoding neurofibromin, p53 and p16 (19). S462 showed a dose-dependent reduction of cell growth *in vitro* (Figure 4A). Ligand induced phosphorylation of PDGFRα was completely inhibited at both concentrations of imatinib (Figure 4B). The effect of imatinib on PDGFRα positive tumour cells has recently been shown in ovarian cancer cells. Only PDGFRα positive cell cultures were inhibited while cell lines lacking PDGFRα expression were not affected (36). In patients, a mean plasma concentration of up to 4.6 μM imatinib after oral administration of 400 mg (1.46 μM after 24 h) has been reported (17). Therefore, the concentration of imatinib inhibiting MPNST cell culture is similar to that in patient plasma. Further support for the potential sensitivity to imatinib is derived from the observation, that a majority of MPNST patients with *PDGFRA* amplification also exhibit amplification of *KIT*, although the latter seems not to be strongly expressed in tumour cells. However, c-Kit positive mast cells within the tumour are thought to contribute to its development (37). Therefore, MPNST carry at least two targets of imatinib. These data may indicate that patients with MPNST benefit from imatinib treatment.

In conclusion, we describe frequent mutations of *PDGFRA* in MPNST often associated with coamplification of *KIT*. *In vitro* growth of an MPNST cell line could be inhibited by the tyrosin kinase inhibitor imatinib which is known to target both PDGFRα and c-Kit. PDGFRα should therefore be considered as candidate for targeted therapy of MPNST.

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